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Study of the potential therapeutic effect of the mesenchymal stem cells (MSCs) secretome on mammary cancer cells

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Resumo

As células estaminais, detentoras de características únicas, têm vindo a despertar um interesse cada vez maior na investigação científica, com um potencial biológico que as torna em alvos cada vez mais apetecíveis no desenvolvimento de terapias inovadoras.

Para serem definidas como tal as células estaminais têm que obedecer a três parâmetros (1). O primeiro consiste na capacidade para se autorrenovarem ao longo da vida, nomeadamente através de um processo de reprodução assimétrica, originando, por um lado, uma célula indiferenciada, perpetuando assim o seu carácter estaminal, e dando origem, por outro, a uma célula que se diferenciará num tipo específico (1–3). O segundo parâmetro consiste na capacidade que estas células possuem de se diferenciar num tipo celular específico, sendo que aqui a plasticidade das mesmas está dependente do seu estadio de potência (1). Finalmente, a definição de estaminalidade dita que estas células deverão ser capazes de repovoar o seu tecido residente em caso de lesão. É importante realçar que estas células estão presentes em nichos em todos os tecidos, embora a sua taxa de renovação seja muito variável no prisma inter-tecidual (1,4,5).

O potencial de diferenciação de uma célula estaminal vai afunilando com o avanço do desenvolvimento embrionário (6). Após a fertilização, uma célula diz-se totipotente, o estado mais basal do carácter estaminal, sendo capaz de se diferenciar em qualquer tipo celular no organismo, embrionário e extra-embrionário. Aquando da formação do blastocisto, as células adquirem um carácter pluripotente, sendo comummente designadas como células estaminais embrionárias (CEE), já sem a capacidade de originar tecidos extra-embrionários mas sendo ainda capazes de originar tecidos dos três folhetos embrionários na altura da gastrulação. Com o progresso do desenvolvimento embrionário, as células sofrem ainda outra redução no seu potencial de diferenciação, designando-se neste estado como multipotentes (1,6).

Contudo, existem ainda evidências de plasticidade em algumas células estaminais adultas, nas quais se incluem as células estaminais mesenquimatosas (CEM) (7,8). Estas células podem ser isoladas de vários tecidos neonatais como a placenta, o saco amniótico (9,10), sangue (11) e cordão umbilical (12), e adultos como a medula óssea, o fígado (13) e o tecido adiposo (14).

Segundo as regras estabelecidas pela Sociedade Internacional para a Terapia Celular, as CEM têm que obedecer a três parâmetros: 1) capacidade de aderência a superfícies de plástico quando em condições normais de cultura celular; 2) expressão de antigénios específicos de superfície como CD105, CD73 e CD90, e não expressão de antigénios embrionários específicos entre os quais CD45, CD34, CD14, CD11b, CD79a, CD19 e HLA classe II; 3) capacidade de diferenciação em adipócitos, osteoblastos e condrócitos, quando em condições de cultura apropriadas para o efeito (15).

Em comparação com as CEE às quais são inerentes vários constrangimentos éticos derivados da manipulação de embriões humanos, bem como às células pluripotentes induzidas (16), que apresentam uma elevada instabilidade genética (17), as CEM apresentam-se muito atrativas do ponto de vista da sua utilização e aplicação, por apresentarem métodos de isolamento fáceis e não invasivos. Mais ainda, as CEM demonstram uma grande capacidade replicativa *in vitro*, que constitui um fator preponderante tendo em conta o elevado número de células normalmente requerido para procedimentos de terapia celular (1).

As CEM têm suscitado um grande interesse do ponto de vista terapêutico, entre os quais ao nível da medicina regenerativa e toxicologia *in vitro*. Uma das principais características das CEM é a sua capacidade intrínseca de migrar para locais de inflamação, geralmente como resposta a redes de

sinalização de citoquinas e/ou fatores de crescimento (18). Dotadas desta habilidade, as CEM chegam ao tecido/região lesada e aí promovem a regeneração tecidual quer através de mecanismos de diferenciação quer através da secreção de fatores parácrinos (18). Outra característica única das CEM é a sua capacidade para modular o sistema imunitário, tendo inclusivamente sido empregues na clínica ao nível, por exemplo, da "graft versus host disease", na qual as células transplantadas promovem uma reação imune contra o organismo recipiente. As CEM, ao modularem a regulação de células do sistema imunitário (B, T, Linfócitos Natural Killer), permitem contornar este obstáculo inerente a várias doenças auto imunes (19).

Curiosamente, grande parte dos efeitos terapêuticos inerentes às CEM advêm não da entidade celular por si só, ou de efeitos de diferenciação, mas sim dos fatores por elas produzidos, fatores parácrinos, comummente designados como secretoma, constituído por elevadas quantidades de proteína nomeadamente citocinas e fatores de crescimento (20). Este secretoma das CEM já foi responsável por efeitos de regeneração em vários modelos de doença, nomeadamente acidente cardiovascular, enfarte e regeneração de menisco (21).

Representando entidades cada vez mais atrativas do ponto de vista da investigação científica, vários tipos de cultura celular têm sido desenvolvidos e otimizados ao longo dos últimos anos, na tentativa de maximizar o potencial terapêutico das CEM. Entre estes, a perspetiva de culturas tridimensionais das CEM tem vindo a crescer em interesse, apresentando mudanças na arquitetura celular que possibilitam uma maior aproximação àquele que é o ambiente que as células encontram em condições *in vivo*, em comparação com as culturas celulares em monocamada, tradicionais, as quais quando prolongadas estão associadas à perda de características celulares especificas (22). Quando cultivadas em 3D, as CEM apresentam tanto uma capacidade de diferenciação aumentada, como um secretoma mais rico, que se traduz, entre outros, num aumento das suas propriedades anti-inflamatórias, angiogénicas e de regeneração (22).

Além do seu papel já provado em cenário de ferida e regeneração, a sua capacidade de migração para tumores faz ainda das CEM potenciais alvos terapêuticos para o cancro. Contudo, o seu papel neste âmbito tem-se pautado maioritariamente por trabalhos contraditórios, contrapondo estudos que evidenciam um papel anti-tumoral das CEM a outros que constatam precisamente o oposto, sendo este um tópico alvo de grande discussão na comunidade científica atual (23,24).

Assim, o principal propósito deste trabalho foi avaliar o efeito do secretoma das CEM como possível terapêutica para o caso de cancro da mama. CEM neonatais humanas (CEMnh) isoladas a partir do cordão umbilical (UCX®; ECBio, PCT/IB08/54067) foram usadas para este propósito, visto terem já provado efeitos benéficos noutro tipo de doença ou lesão. Deste modo, o secretoma destas células foi produzido tanto em culturas tradicionais 2D, como em 3D, sob a forma de meio condicionado (CM2D/CM3D). Para a produção de meio condicionado 3D (CM3D), as CEM foram cultivadas em sistemas de cultura em suspensão agitados, e sujeitas a um período gradual de redução de soro, recorrendo a um protocolo previamente otimizado. As células cultivadas nesta condição formaram agregados cuja morfologia e diâmetro (< 250 µm) não sofreram alterações de maior ao longo de todo o protocolo, garantindo assim uma cultura viável e sem a presença de centros necróticos, da qual no fim foi recolhido o CM3D.

Após a produção do CM2D/3D, o seu efeito foi avaliado, em primeira instância, na viabilidade de duas linhas celulares mamárias humanas: uma linha não tumoral (MCF10A) e uma linha tumoral invasiva (MDA-MB-231). Este efeito do CM (1x e 10x concentrado) foi avaliado isoladamente, e em combinação com o fármaco quimioterapêutico doxorrubicina (dox: 100 nM a 1000 nM), que constitui a primeira linha de terapêutica farmacológica contra o cancro da mama. No geral, para a linha não

tumoral, verificou-se um efeito protetor significativo de ambos os CM contra a citotoxicidade induzida pela dox, particularmente no caso do CM2D/CM3D 10x concentrados. Mais ainda, esta proteção apresentou uma magnitude mais elevada no caso do CM3D (1.5x mais) quando comparado com o CM2D. Para a linha tumoral, nenhum efeito significativo adveio da aplicação do CM, apresentando-se apenas um ligeiro aumento de viabilidade celular no tratamento combinado do CM com a dox, em comparação com o fármaco sozinho.

Em seguida, o efeito do CM2D/3D (1x e 10x), sozinho e em combinação com a dox (100 nM), foi avaliado na capacidade migratória da linha celular tumoral, através de um ensaio de ferida *in vitro*. Neste ensaio, os resultados apontaram para um aumento significativo da capacidade migratória das células tumorais, tanto no caso do CM2D como do CM3D, especialmente no tratamento com a concentração 10x.

Por fim foi ainda avaliada a capacidade de invasão da linha celular tumoral através de um ensaio de quimiotaxia "transwell", nas mesmas condições de tratamento empregues no ensaio de migração. Aqui, apesar de nenhum resultado obtido possuir significado estatístico, é possível observar a promoção de duas tendências opostas, consoante o tipo de tratamento empregue. Quando administrado sozinho, o CM manifestou uma ligeira tendência para promover a capacidade invasiva das células (10x para o CM2D, 1x e 10x para CM3D), enquanto que em combinação com a dox, o contrário foi observado. A dox, por si só, pareceu provocar um ligeiro decréscimo da capacidade de invasão celular, efeito esse que foi um pouco mais pronunciado no caso do tratamento combinado com o CM2D/3D (10x concentrado).

Em suma, apesar dos resultados obtidos sugerirem alguns efeitos que apontam em diversas direções, evidenciou-se que o CM das CEMnh em estudo possui um papel protetor importante nas células não-tumorais contra a dox, o que constitui um aspeto importante num possível cenário de terapia combinada para cancro da mama, no âmbito de proteção dos tecidos saudáveis adjacentes ao tumor. Mais ainda, este efeito foi mais marcado no CM proveniente das culturas tridimensionais, demonstrando a importância crescente deste tipo de cultura em cenários inovadores de terapia celular. Contudo, mais estudos deverão ser feitos para clarificar a segurança no uso do CM das CEM para terapia em cancro da mama.

Palavras-Chave

Células estaminais mesenquimatosas neonatais humanas da matriz do cordão umbilical, culturas 3D, secretoma, meio condicionado, cancro da mama

Abstract

Breast cancer remains one of the leading causes of tumour-related death in women, mainly due to the development of metastasis at distant sites from the primary tumour. In addition to the clinically heterogeneity of primary tumours, the heterogeneous metastatic pattern usually impairs the efficacy of the current treatments contributing to the poor prognosis of advanced breast cancer.
Mesenchymal Stem Cells (MSCs) have demonstrated an inherent homing ability towards tumours that makes it an attractive novel field of research in the context of cancer therapy. However, MSCs impact in cancer is still controversial, with literature reporting both cancer promoting and inhibitory properties. Additionally, three-dimensional (3D) cell cultures have been reported as more *in vivo-like*, in aspects of morphology, viability and functionality, presenting also an enhanced paracrine activity with beneficial effects, for instance, in tissue regeneration.
This work aimed to investigate the effect of the secretome of UCX®, a specific population of human neonatal umbilical cord matrix derived MSCs (hnMSCs), on human breast cancer. Conditioned medium from UCX® (MSCs-CM) was produced and collected from both 2D monolayer cultures (CM2D) and 3D dynamic spinner flask suspension cultures (CM3D).
Cell viability/proliferation under treatment with UCX®-CM (1x and 10x concentrated) either alone or in combination with the chemotherapeutic drug Doxorubicin (Dox, 100-1000 μM) was evaluated through MTS assay in two human breast cell lines: MDA-MB-231 (malignant; invasive) and MCF10A (non-malignant epithelial cells). In MDA-MB-231 cells, a slight increase in cancer cell viability in the presence of Dox (n.s.) and CM2D or CM3D was observed; while for the MCF10A cell line both CM2D 10x ($p<0.05$) and CM3D ($p<0.001$) significantly decreased the cytotoxicity induced by Dox at 0.1 μM.
Additionally, breast cancer cells' ability to migrate was evaluated through an *in vitro* scratch assay when treated with CM2D/CM3D (1x and 10x), alone and in combination with Dox (0.1 μM). Herein, a significant increase in MDA-MB-231 cell capacity to migrate when treated with both CM2D and CM3D 10x ($p<0.05$) was observed, independently of Dox treatment.
Finally, breast cancer cells capacity to invade tissues was also assessed through a coated-transwell chemotaxis assay, under the same conditions applied for the scratch assay. Results showed that, although non-significantly, both CM2D (10x) and CM3D (1x and 10x) promoted cell invasion (n.s.). Dox, on the other hand, showed a trend to reduce cell invasion ability, an effect slightly enhanced in the combined treatment with CM2D and CM3D (10x) (n.s.).
In sum, although showing a trend for promoting breast cancer cells features when administered alone, the combined treatment of UCX®-CM with Dox attenuated Dox cytotoxicity in the non-tumoral cells and decreased breast cancer cell invasion capacity. Therefore, further studies are needed to better understand the underlying mechanisms of UCX®-CM in breast cancer.

Keywords

Umbilical cord tissue-derived human neonatal mesenchymal stromal cells, 3D cultures, secretome, conditioned medium, breast cancer

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List of Abbreviations

2D – Two-dimensional
3D – Three-dimensional
AT-MSCs – Adipose Tissue derived Mesenchymal Stem Cell(s)
ATMP – Advanced Therapy Medicinal Product
BAX – Bcl-2 associated X protein
Bcl-2 – B-cell lymphoma 2
BDNF – Brain Derived Neurotrophic Factor
bFGF – basic Fibroblast Growth Factor
BM – Bone Marrow
BM-MSC(s) – Bone Marrow derived Mesenchymal Stem Cell(s)
BxPC – Pancreatic adenocarcinoma cell line
CM – Conditioned Medium
CM2D – Conditioned medium derived from UCX® cultured in monolayer
CM3D – Conditioned Medium derived from UCX® cultured in 3D spheroids
CSCs – Cancer Stem Cells
DKK-1 – Dickkopf-1 factor
DMEM – Dulbecco's Modified Eagle's Medium
DNA – Deoxyribonucleic Acid
Dox – Dox
EDTA – Ethylenediamine Tetraacetic Acid
EGF – Epidermal Growth Factor
EMT – Epithelial to Mesenchymal Transition
EPC(s) – Endothelial Progenitor Cell(s)
ESC(s) – Embryonic Stem Cell(s)
FBS – Fetal Bovine Serum
HGF – Hepatocyte Growth Factor
hnMSC(s) – human neonatal Mesenchymal Stem Cell(s)
hUCMSC(s) – human Umbilical Cord Mesenchymal Stem Cell(s)
hUCESC(s) – human Uterine Cervix Mesenchymal Stem Cell(s)
IFN-γ – Interferon gamma
IGF-1 – Insulin-like Growth Factor-1
IL-1α – Interleukin-1 alpha
IL-1β – Interleukin-1 beta
IL-6 – Interleukin-6
IL-8 – Interleukin-8
IL-10 – Interleukin-10
IPSC(s) – Induced Pluripotent Stem Cell(s)
ISCT – International Society for Cell Therapy
KGF – Keratinocyte Growth Factor
MDSC(s) – Myeloid Derived Suppressor Cells
MHC – Major Histocompatibility Complex
MMP(s) – Matrix Metalloproteinase(s)
MS – Multiple Sclerosis
MSCs – Mesenchymal Stem Cell(s)
MTS – (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)
PBS – Phosphatase Buffered Saline
PDGF – Platelet-Derived Growth Factor
PTEN – Phosphatase and Tensin Homologue
PTX – Paclitaxel

ROS – Reactive Oxygen Species
SD – Standard Deviation
SDF-1 – Stromal Cell Derived Factor 1
SERPINE 1 – Serpin Family E Member 1
SFSC – Spinner Flask Suspension Culture
Slug – Zinc finger protein SNAI2
Snail – Zinc finger protein SNAI1
TAF(s) – Tumour Associated Fibroblast(s)
TGF-β – Transforming Growth Factor Beta
TLR – Toll-like Receptor
TME – Tumour Microenvironment
TNF-α – Tumour Necrosis Factor Alpha
VEGF – Vascular Endothelial Growth Factor
WJSC(s) – Wharton Jelly's Derived Stem Cell(s)
XIAP – X-linked Inhibition of Apoptosis Protein
ZEB1 – Zinc Finger E-Box Binding Homeobox 1
α-MEM – Minimum Essential Eagle Medium Alpha Modification

1. Introduction

1.1. Stem Cells

It was early in 1963, that McCulloch and Till first reported the discovery of undifferentiated, self-renewing, progenitor cell populations, nowadays termed as stem cells (25). Horizons were set to an all new perspective of stem cell based therapy, and thus, since then, stem cell field of research has been growing exponentially. This has been accompanied by a further advance in the knowledge of biological and functional features of these cells, which became an ever-growing target of interest regarding biomedicine.

Stem cells are defined as such if matching three criteria (1). Firstly, they must possess the ability to self-renew throughout their life and to preserve their stem-cell like features. This is accomplished through a process named asymmetric division, in which a stem cell gives origin to two other cells, one maintaining its original stemness, and another to be differentiated into a specific cell type (1–3).
Thereafter, these cells are secondly characterized by their ability to differentiate into a specific type of cell, acquiring morphology, phenotype, and physiological properties that categorize it as belonging to a particular tissue (1).
The third and last required stem cell-criterion is the ability to renew the tissues it populates. This situation is quite variable, however, according to the body region to which it is concerned. Blood-forming stem cells, gut epithelium stem cells, and skin forming stem cells are constantly replaced for normal health, for instance, as opposed to what happens in the nervous system, in which stem cells possess a rather quiescent character.

Stem cells are dispersed in microenvironments throughout the body, coined by Schofield as “niches”, composed of tissue cells and extracellular substrates that can indefinitely house and regulate stem cells through secretion of growth and angiogenic factors. (1,4,5)
Is noteworthy that a cell stemness potential narrows over the process of embryonic development, decreasing its differentiation potential as they become more and more compromised with a specific tissue derived from one of the germ layers (6). After fertilization, cells possess a totipotent character, the most basal degree of stemness, being able to undergo differentiation onto any cell type of the organism, both embryonic or extra-embryonic. Further ahead in the differentiation stage, by the time the blastocyst is formed, the differentiation potential of these cells is narrowed to what is known as the pluripotent state of cells. Pluripotent cells, present in the inner cell mass of the embryo, conversely to totipotent ones, lack the ability to form extra-embryonic tissues, but can still differentiate into any cell type of the three embryonic germ layers – endoderm, mesoderm and ectoderm – by the time of gastrulation. One example of pluripotent stem cells is embryonic stem cells (ESCs). With the progress of embryonic development, cells’ differentiation potential continues to narrow down, becoming constricted into only two of the three germ layers. At this stage, cells are designated as multipotent. As tissues are progressively formed in development, almost all cells of the embryo have begun to specialize into specific tissues (1,3). Therefore, after birth, although not disappearing, stem cells have less specificity, less potential, and are more constricted concerning their differentiation potential.

Nevertheless, certain post-natal stem cells, in which mesenchymal stem cells are included, have been shown to be able to differentiate into other cell types than their tissue of origin, under certain conditions, showing possible evidence of a certain plasticity and ability for cellular trans-differentiation (7,8).

Stem cell road towards therapeutic application has not been a straightforward one. The possible usage of ESCs in therapy was never consensual, despite their pluripotent character, due to the ethical problems associated with the handling of human embryos, as well as the problem of tissue rejection after transplantation in patients. Furthermore, under *in vitro* culture conditions, human embryonic stem cells (ESCs) have been reported to become aneuploid, i.e., they acquire an abnormal number of chromosomes (17). Thus, Yamanaka proposed in 2006, in his Nobel awarded study, a novel alternative consisting on the use of the nowadays known induced Pluripotent Stem Cells (iPSCs), in order to overcome constraining issues imposed by the use of ESCs (16). However, even iPSCs imply some constrains when regarded as models for disease and transplantation therapies. There are three major issues regarding iPSCs suitability for use: 1) iPSCs display more genetic and epigenetic abnormalities than do ESCs or fibroblasts, from which they are originated. They also present an early appearance of chromosomal abnormalities when comparing to ESCs, with an estimated ten times higher frequency of mutations comparing to the latter; 2) Emergence of abnormalities at different stages of iPSCs generation. These can either come from the cell from which they were reprogrammed, as well as from consequences associated to long-term adaptation to cell culture, and include, for instance, an over-representation of the short arm of a chromosome, an entire chromosome, and a sub-region in the long arm of a chromosome; 3) iPSCs, as ESCs, display an enrichment in regions prone to amplification, deletion or point mutation in genes often involved in cell cycle regulation and cancer (17).

In light of the disadvantages related to both ESCs and iPSCs mentioned above, the interest concerning the use of Mesenchymal Stem Cells (MSCs) for therapeutic scopes still persists. MSCs present easy and non-invasive isolation procedures, unlike ESCs, and dodge the genetic abnormalities associated with the process of reprograming and culture of iPSCs. This allied to a remarkable replicative ability *in vitro*, which is of extreme importance having in account the large number of cells usually needed for cell therapy purposes, makes MSCs a major target of interest in cell therapies (1).

1.2. Mesenchymal Stem/Stromal Cells

Mesenchymal Stem Cells (MSCs), a term coined in 1991 by Caplan (26), were first described by Friedenstein et al., (27). These cells englobe a division of less committed post-natal stem cells, that are present and can be isolated from various adult tissues such as bone marrow (BM), thymus, brain, liver, lung, kidney, aorta, muscle, spleen (13) and adipose tissue (14), as well as from neonatal ones including amniotic sac and fluid, placenta (9,10) umbilical cord blood and tissue (12), or even foetal blood, liver and bone marrow (11).

Inconsistencies in the literature regarding MSCs characterization dictated the establishment of a standard set of criteria by the International Society for Cell Therapy (ISCT), according to which, in order to be classified as such, a cell must possess: 1) plastic-adherence ability when in normal culture conditions; 2) over 95% expression of specific surface antigens such as CD105, CD73 and CD90, and less than 2% expression of embryonic specific stage markers CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA class II; 3) ability to differentiate into osteoblasts, chondroblasts and adipocytes when in proper differentiation conditions (15).

1.2.1. MSCs therapeutic role

MSCs have been generating a growing interest regarding their application in a wide variety of fields, of which stand out regenerative medicine and *in vitro* toxicology. MSCs have been described as playing

an important role in several organism phenomena (Fig. 1.1.), due to several mechanisms inherent to these cells, namely: 1) homing ability towards sites of inflammation after tissue injury; 2) ability to differentiate into various cell types; 3) ability to secrete multiple bioactive molecules capable of stimulating recovery of injured cells and inhibiting inflammation; 4) lack of immunogenicity and the ability to perform immunomodulatory functions (18).

MSCs inherent homing ability is sustained as a response to a multitude of signals, including growth factors such as insulin like growth factor 1 (ILGF1) and platelet-derived growth factor (PDGF) (18), while CXCL12 and CX3CL cytokines, for instance, were also reported as able to induce migration of MSCs expressing their receptors, CXCR4 and CX3CR1 respectively (28). This migration behaviour can also be regulated by other factors: Basic Fibroblast Growth Factor (bFGF), depending on its concentration, was shown to induce opposite effects on MSCs, as low concentrations of this molecule led to cell attraction, whereas higher concentrations resulted in its repulsion (25,26); Matrix Metalloproteinases (MMPs), namely MMP-2, were also reported as playing a part in the extent of MSCs migration, being highly expressed in these cells during their homing process (29,31); Toll Like Receptor (TLR) signalling also provides input for migration to occur on these cells, as shown in Tomchuck's work (29,32), in which MSCs migration promoted by TLR was inhibited by TLR neutralizing antibody.

Endowed with an intrinsic homing ability towards injury sites, MSCs will therein display a regenerative effect, establishing cell-cell connections, reducing inflammation, apoptosis and fibrosis of damaged tissues, and stimulating tissue regeneration (18). Indeed, MSCs anti-inflammatory and regenerative properties have proved useful in clinical trials, for instance, in multiple sclerosis (MS), where patients injected with human Umbilical Cord MSCs (UC-MSCs) experimented alleviation of clinical symptoms, with a following significant improve in the Expanded Disability Status Scale score (33).
MSCs immunomodulation capacity is reflected in very low expression levels of major histocompatibility complex (MHC) Class I antigens and no expression of MHC Class II antigens and molecules such as CD40, CD80, and CD86. This was reported to allow Bone Marrow MSCs (BM-MSCs) to evade the organism's immune response, through the inhibition of proinflammatory cytokines, such as tumour necrosis factor-α (TNF-α) and interferon gamma (IFN-γ), and overexpression of suppressive cytokines, including interleukin-10 (IL-10), leading to suppression of immune agents T- and B-cells, and inhibiting proliferation and cytotoxicity of natural killer (NK) cells as well (18). Le Blanc et al. (34) provided therapeutic proof of the immunomodulatory character of MSCs, concerning Graft versus Host Disease (GvHD). GvHD occurs in transplants of haematopoietic cells, for instance, in which the transplanted cells formulate an immune response against the transplant recipient. In the cited study, based on a novel therapeutic approach, injection of bone marrow derived MSCs (BM-MSCs) onto a patient ultimately resulted in the treatment of its grade IV acute GvHD, reducing diarrhoea frequency, abolishing abdominal pain, and causing regain of appetite. However, the safety usage of MSCs regarding transplant-related therapies is not yet certain. A pilot clinical study conducted by Chen et al. (35), showed that patients co-transplanted with MSCs, despite showing a lower incidence of GvHD, manifested a significant higher recurrence rate, when compared to patients not treated with MSCs.

1.2.1.1 Paracrine Effects

MSC abilities go beyond mere stimuli-response and differentiation capacity. Although it was firstly thought that the cell entity itself was the main responsible for its therapeutic effects, it is accepted nowadays that the major success behind MSCs therapeutic scopes, including tissue repair, immunomodulation, etc., is related to their paracrine activity, in the form of the secretome. MSCs secretome is a rich source of proteins including cytokines, chemokines and growth factors, that has gained great attention during the last years because of its multiple implications in regenerative medicine (20). Evidence of MSCs' capacity to secrete a wide array of factors dates back to at least twenty years ago (36). As MSCs progress towards a more differentiated phenotype, the quantity and array of secreted bioactive factors changes as their descendants enter new lineage stages, functioning this pattern of secreted factors as a feedback on the cell itself, allowing it to regulate both its functional status and physiology [22]. Importantly, a considerable part of MSCs regenerative properties mentioned in the section above, such as reduction of inflammation and tissue regeneration, are processes sustained by the secretion of several molecules, including cytokines such as transforming growth factor beta (TGF-β), IL-10, and IL-6, as well as growth factors such as vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), keratinocyte growth factor (KGF), among others (18). A very interesting review by Caplan and Dennis mentions several MSCs mediated effects in various illnesses, which occurred without the need for local engraftment (21). In a stroke (brain) scenario for instance, MSCs did not differentiate into neurons or neuronal support cells, but instead supplied several bioactive agents that inhibited scar formation, inhibited apoptosis, increased angiogenesis and stimulated the action of intrinsic neural progenitor cells to regenerate functional neurological pathways (synaptogenesis, neurogenesis), regaining coordinated function (21). In another work, through production of brain derived neurotrophic factor (BDNF), insulin-like growth factor-1 (ILGF-1) and neurotrophin-3 (NT-3), MSCs cells could regulate compensatory responses during both acute and chronic strokes. This was achieved by inducing the proliferation of inherent cells, reducing apoptosis of damaged cells, and promoting angiogenesis, axonal sprouting and synaptogenesis (33). Similarly, MSCs displayed regenerative effects regarding infarct (injured heart), by secreting factors that induced neo-vascularization, inhibition of scarring, decreased cardiomyocyte apoptosis and increased nerve sprouting. Finally, this throphically enhanced regeneration was also achieved in a meniscus model, with MSCs inhibition of scarring and apoptosis at the cut surface, stimulating angiogenesis and proliferation of the host-derived reparative cells to fabricate a new meniscus (21). In a more recent work, MSCs secretome also proven itself effective in the treatment of chronic massive rotator cuff tears, by augmenting tendon cell viability *in vitro*, which enhanced tendon-bone healing *in vivo* (37). Additionally, a work developed in our laboratory contributed to further sustainment of the therapeutic properties MSCs secretome, showing a complete regeneration outcome in an *in vivo* skin wound healing model (38).

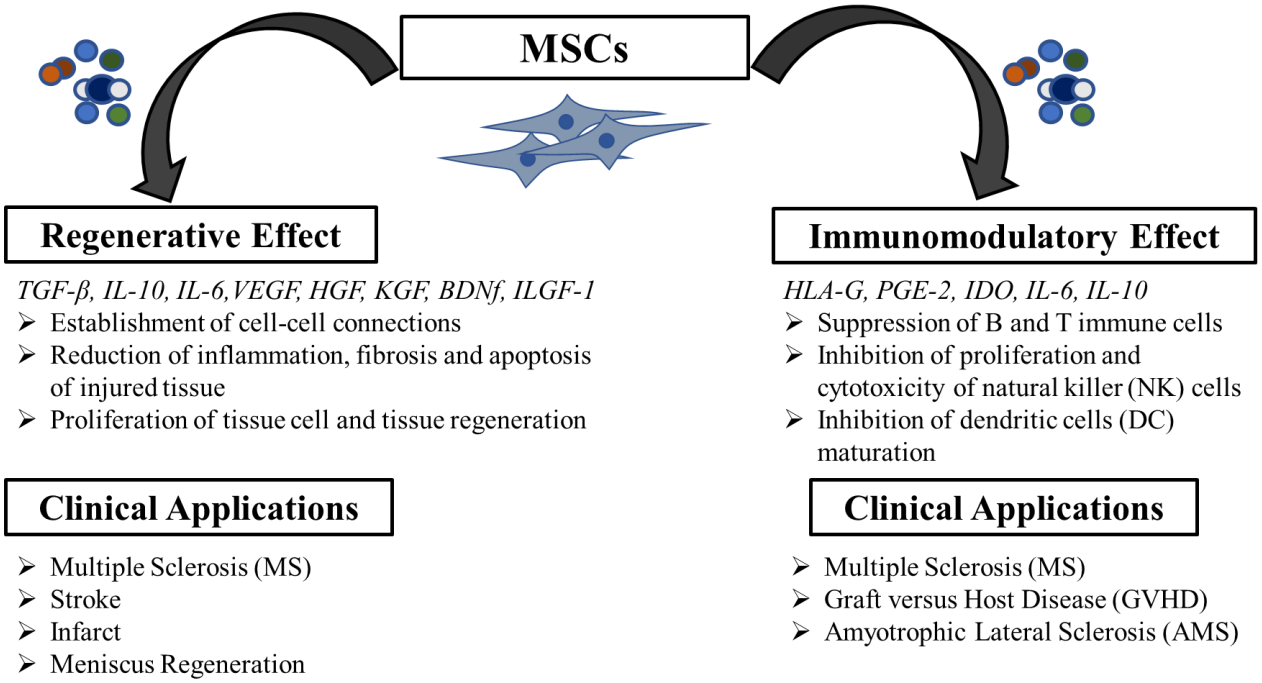

Figure 1.1. – Mesenchymal Stem Cells (MSCs) regenerative and immunomodulatory properties as a therapeutic agent

1.2.2. 3D Cultures

Revealing itself as a promising therapeutic asset, several strategies, such as three-dimensional (3D) cultures, have been attempted to improve MSCs culturing process. Although initially explored for embryonic and malignant cells, nowadays the potential of 3D culture is growing at a very fast pace, being explored in various biomedical research perspectives, such as stem cell biology (39), development of more suitable *in vitro* cancer models (40,41) and hepatic models for drug testing (42). Importantly, 3D cultures are one of the core focus of our group of investigation. Indeed, in a recent work, we were able to achieve a competent development of 3D hepatocyte spheroid models, as a bridge to enable the identification of possible biomarkers of bioactivation/toxicity that constitute an upstream resource for the pharmaceutical industry, flagging drug safety alerts in early stages of drug development (43). Additionally, parallel work is also being currently done in developing 3D models of breast cancer also for drug testing. In fact, the migration capacity of breast cancer spheroids is differently affected by inhibitory agents when compared to monolayers, evidencing the importance of having more *in vivo like* models in order to properly assess cancer therapy.

MSCs cultured in the form of spheroids were reported to better resemble the cell environment *in vivo*, with changes in cellular architecture that enable greater cell–cell and cell–matrix interactions when compared to 2D cultures that when prolonged are more susceptible to the loss of cell-specific properties (22). Additionally, MSCs cultured in 3D aggregates present a more physiological structure regarding cell morphology, viability and functionality. The gene expression profile itself also presents considerable changes between the two culture types. While genes associated with extracellular regions, regulation of cell adhesion, receptor binding, cell communication and inflammation sustained higher expression in 3D cultured MSCs, genes encoding for cytoskeletal molecules, biogenesis, mitosis and

cell cycle were decreased when compared with 2D cultures. 3D-MSCs up-regulated genes include pro-inflammatory cytokines, IL-1α, IL-1β, IL-6, IL-8; anti-inflammatory genes such as leukemia inhibitory factor (LIF), stanniocalcin-1 (STC-1), prostaglandin E2 (PGE-2); and several angiogenic factors including angiogenin, VEGF or proliferative/migratory factors such as FGF and HGF. Therefore, several biological properties such as angiogenic, anti-inflammatory, and regenerative effects, as well as survival after transplantation are upgraded in 3D cultures of MSCs (22).
Of note, the skin wound healing study conducted in our laboratory mentioned in the "paracrine effects" section above further reported that MSCs cultured under 3D conditions showed increased paracrine activity, with expression of higher levels of growth factors such as VEGF, MMP-2, MMP-9 and HGF, involved in tissue regeneration and healing. Hence, treatment of skin wounds with conditioned medium produced from MSCs cultured in spheroids, resulted in a significant better and faster healing outcome, when compared to administration of conditioned medium produced by MSCs in two-dimensional culture conditions (38). In a similar work, MSCs cultured as 3D cell aggregates significantly increased the rate of wound closure in a diabetic wound healing mouse model, sustained by enhanced expression of extracellular matrix proteins (tenascin C, collagen VI a3, and fibronectin) and secreted soluble factors (HGF, MMP-2 and MMP-14) when compared to traditionally cultured MSCs (44). Hence, 3D cultures of MSCs appear to modulate MSC trophic factor secretion to be ultimately used in biomedicine approaches.

1.3. Breast Cancer

Regarded as a major world epidemy, an increasing scientific endeavour has been made in the past few years to better try to understand and mitigate cancer impact. Risk factors for cancer can vary widely across different environments. In western developed countries in general, risk behaviours such as smoking, dietary patterns and reproductive behaviours are the most prevalent, while in economically developing countries infectious agents remain the most significant (45). The process of tumour development, is known to result, in part, from the interaction between tumour cells and their surrounding supporting tissues, in which mutual interactions of tumour cells and stromal cells affect neoplastic cell growth, neo-angiogenesis, and extracellular remodeling. (46–48).

Breast cancer, in particular, was considered in 2012 to be the 2nd most diagnosed cancer and the 5th leading cause of dead by cancer in the world (49). Breast cancer lethality in developed countries has been diminishing, due to the improvement of detection methods, allowing for earlier diagnosis, preventing the progression of the tumour and its consequent metastization. Currently, main clinical strategies regarding breast cancer are based in surgery, radiotherapy and chemotherapy treatments, which are effective to a certain extent, but can also be associated with side effects (50,51). Anticancer drug mechanisms depend mainly on their ability to damage the DNA, generating lesions that ultimately induce cell death. Cancer cells are, however, often resistant to DNA-damaging agents, due to a set of different mechanisms, including increased activity of DNA repair pathways (52).

Doxorubicin (dox), an anthracycline antibiotic, is a first line chemotherapeutic agent against breast cancer. The antitumor activity of dox is associated with its ability to bind to DNA-associated enzymes, intercalate with DNA base pairs, and target multiple molecular pathways to produce a range of cytotoxic effects, such as the activation of protein kinase (AMPK) via reactive oxygen species (ROS)-dependent liver kinaseB1 (LKB1). This mechanism provides the upstream signal necessary for AMPK activation, resulting in p53 up-regulation, which initiates apoptosis in cancer cells (53). P53 tumour suppressor protein is one of the most important cell cycle controllers in normal cells, initiator of apoptosis to

eradicate damaged cells, and its expression is usually hampered in cancer scenarios, reducing cancer cell apoptotic death. Dox, by re-activating p53, will therefore promote apoptosis in cancer cells (54). Dox DNA-damaging effects can also be a consequence of poisoning topoisomerase IIα, DNA binding and alkylation, establishment of DNA inter-strand crosslinks, and formation of base damage and strand breaks induced by the generation of reactive oxygen species (ROS) (52).

However, this drug also presents cytotoxic effects in off target cells from the patients. It not only is cytotoxic to non-tumoral cells, but is also associated to cardiotoxicity and heart failure (55). Other effects often consist on depression of the immune system, decrease in number of immune cells, increasing patient's susceptibility to microbial infections, fatigue and decrease in healing time. The severity of these effects is linked to the dosage of dox administrated, and to the regeneration capacity of the patient's bone marrow (56).

1.3.1. MSCs in cancer

Apart from their role in wound healing and immunomodulation processes, MSCs have also been regarded as possible cancer therapeutic agents, since they exhibit an intrinsic ability to migrate towards tumours. In the pro-inflammatory tumour microenvironment (TME) the expression of an array of chemokines and cytokines occurs, namely VEGF, FGF, PDGF, CCL5 and IL-8, that cause MSCs to migrate toward these tissues in a stepwise manner by creating a chemokine concentration gradient (57). However, the role of these cells in tumorigenesis is still quite controversial. Some studies report a pro-tumorigenic role, with enhancement of tumours and its features when exposed to the action of MSCs, while others describe the latter as tumour inhibiting agents, both *in vitro* and *in vivo* (Fig. 1.2.). Thus it is not clear whether the clinical application of MSCs will eventually lead to unforeseen and unwanted side effects (23,24). Below are described some examples of the discrepant roles of MSCs reported in the literature.

1.3.1.1. Pro-tumour role of MSCs

In the extent of cancer promotion, several studies account for the participation of MSCs in intensification of breast cancer features, promoting cancer cell proliferation and protection from apoptosis, for example.

Fierro and colleagues (58), showed that after co-culturing MCF-7 breast cancer cells with BM-MSCs, changes in the phenotype of cancer cells were observed, with enhanced nucleus size, increased proliferative ability, and a decrease in E-cadherin expression. These alterations led to an induction of epithelial to mesenchymal transition (EMT), suggesting a pre-disposition of cancer cells towards a more invasive phenotype. By testing the isolated action of VEGF and IL-6 factors over MCF-7 cells, results obtained were much similar, compared to the ones from BM-MSCs, hence implying these factors on the MSCs cancer promoting role.
Co-cultures of MCF-7 and MDA-MB-231 breast cancer cell lines with MSCs obtained from breast and abdominal adipose tissue have also been reported to augment cancer cell proliferation. Up-regulation of FGF2, Serpin Family E Member 1 (SERPINE1), VEGFA, and VEGFC genes was noted in both types of adipose derived MSCs used in this study, and those genes have been previously associated with progression of breast cancer (59). A similar work, co-culturing breast cancer cells with BM-MSCs

reported a promotion in the proliferation of breast cancer stem cells (BCSCs), generally believed to be the core responsible for tumour progression (60).
Some authors strengthened the pro-tumorigenic role of MSCs derived from the bone marrow (61). By co-administrating BM-MSCs with both MCF-7 and MDA-MB-231 breast cancer cell lines in to immunocompromised mice, an increase in the metastatic potential was observed. Metastasis formation and spread was greater in nude mice co-injected with cancer cells and BM-MSCs when compared to mice injected with cancer cells alone, a mechanism suggested as based in chemokine CCL5. When in contact with breast cancer cells, de novo secretion of this chemokine was promoted in the first, acting then as a promoting cue for motility, invasion, and metastasis.
The predisposition of MSCs to differentiate into cancer associated fibroblasts (CAFs) can also be regarded as a tumour enhancing effect, as these components of the tumour microenvironment (TME) are thought to be correlated with tumour growth, invasion and metastasis. Indeed, human adipose tissue-derived stem cells (ASCs) have been reported to differentiate into a CAF-lyke myofibroblastic phenotype, when treated with breast cancer cell conditioned medium in order to simulate the TME (62).
In a different methodologic approach, the use of conditioned medium from BM-MSCs in cancer cells from mice also increased the migratory capacity in the latter *in vitro* (63). This effect was associated with CXCL1 and CXCL5, highly expressed in the conditioned medium of MSCs, that interacted with CXCR2 positive breast cancer cells, fostering the latter's metastasis to the bone. Muehlberg *et al* (64), also using conditioned medium, this time derived from ASCs, also observed an outcome of tumour promotion in MDA-MB-231 human breast cancer cells and in mouse breast tumour cells 4T1, through the secretion of stromal cell-derived factor-1 (SDF-1).
Finally, a recent study, for instance, reported the ability by MSCs to promote proliferation and inhibit apoptosis of myeloid-derived suppressor cells (MDSC), known for having high immunosuppressive abilities, and usually linked to very bad prognosis in cancer patients. This was accomplished in both *in vitro* direct co-cultures and transwell systems through the up-regulation of Arg1 and NOS2 mRNA and protein levels in MDSC by MSCs, leading to the suppression of T-cell immunity (65); Although not all, some of the mechanisms behind MSCs pro-tumorigenesis effects were already unveiled.
In sum, in the context of enhancing tumorigenic features, MSCs can act by:

1) Suppression of responses from immune system, via secretion of molecules that inhibit proliferation of B, T and nature killer cell activity;
2) Differentiate into cancer-associated fibroblasts (CAFs), a key component of tumour stroma, promoting angiogenesis by recruiting endothelial progenitor cells (EPCs), and directly enhancing tumour growth via secretion of stromal-cell derived factor 1 (SDF-1), acting through the cognate receptor, CXCR4, which is expressed by carcinoma cells (47,66);
3) Contribution to the tumour microenvironment via up-regulation of cancer stem cells (CSCs) proliferation, in a cross-talk mechanism involving IL-6 and CXCL7 factors expressed by cancer cells and MSCs, respectively, triggering CSC proliferative and invasive features (67);
4) Induction of Epithelial to Mesenchymal Transition (EMT), by secretion of molecules such as HGF, EGF, PDGF, and TGF-β, that can activate transcription factors known to promote EMT (Snail, Slug, ZEB1, and TWIST) (68,69);
5) Direct promotion of angiogenesis through secretion of proangiogenic factors such as VEGF and FGF-2, which are enhanced in hypoxic conditions, characteristic of tumours (70);
6) Promotion of tumour metastasis through secretion of factors such as CCL5. Contact between MSCs and tumour cells triggers an enhanced secretion of CCL5 by the first, leading to morphological alterations that induce tumour cell motility, invasiveness, and metastasis, being this mechanism of action also supported by increased activity of matrix metalloproteinases (e.g. MMP-9) secreted by MSC (61,71);

7) Inhibition of cancer cell apoptosis through release of anti-apoptotic/pro-survival factors by MSCs, additionally promoting tumour proliferation, especially in hypoxia conditions (72).

1.3.1.2. Anti-tumour role of MSCs

Parallelly to the works mentioned in the previous section, several other studies performed reported an opposite, cancer hampering effect by MSCs.

Eiró and collaborators (48) studied the effect of the secretome of MSCs derived from normal human uterine cervix (hUCESCs) in three main cell types in breast tumours: cancer cells, fibroblasts and macrophages. Herein, the authors were able to detect, *in vitro*, a significant reduction in cell proliferation through cell cycle arrest and induction of apoptosis, as well a reduced invasion capacity of highly metastatic MDA-MB-231 cell line. In a xenograft *in vivo* mouse tumour model, administration of the same conditioned medium (CM-hUCESCs) resulted in a substantial reduction in tumour growth and increase in overall survival of tumour bearing mice. In addition, targeting CAFs with CM-hUCESCs also resulted in inhibiting the proliferation of these key components of tumour stroma, whereas macrophage differentiation was reverted as well. When trying to ascertain the possible paracrine factors present in CM-hUCESCs that could be involved in tumour inhibition, a 2,5-fold increase in the expression of 4 factors was found, namely TNFSF14, FLT-3, IP-10 and LAP, thus suggesting their implication in cancer suppression mediated by MSCs.

Meleshina and colleagues (73), using BM-MSCs conditioned medium, inhibited the proliferative activity of MDA-MB-231 breast cancer cells *in vitro*. Administration of these cells to an *in vivo* mouse model with pre-established breast tumours resulted in a significant delay and reduction of metastasis formation.

MSCs mediated cancer inhibition through suppression of the Wnt signalling pathway was also reported as correlated with cancer progression (74). Using conditioned medium obtained from human Z3 mesenchymal stem cell line of the dermis tissue, inhibition of MCF7 breast cancer cells growth was observed, through the secretion of dickkopf-1 (Dkk-1) factor, which led to the downregulation of β-catenin and its downstream effects such as c-Myc and Survivin cancer-related genes. Moreover, inhibition of Dkk-1 via small interference RNA attenuated the effects previously described, further strengthening the assumption that Dkk-1 factor secreted by these cells can be involved in cancer inhibition via disruption of the Wnt signalling pathway (75).

Gauthaman and colleagues also observed an anti-tumorigenic effect of MSCs (76). Conditioned medium and cell lysate from Wharton's jelly stem cells (hWJSC) derived from the umbilical cord inhibited MDA-MB-231 cells proliferation *in vitro*, with evidence of cell cycle arrest, inhibition of migration and induction of apoptosis. In fact, MSCs secretome promoted both the up-regulation of pro-apoptotic BAX gene and downregulation of anti-apoptotic BCL2 and SURVIVIN genes. It is noteworthy that in this study, the tumour suppressing effect from hWJSC was not exclusive to breast cancer cells, comprising also ovarian carcinoma (TOV-112D), and osteosarcoma (MG-63) cells. The same group also reported a similar outcome *in vivo*, achieving attenuation of breast adenocarcinoma tumours, in both early and late onset (77).

Ma and colleagues (78), using co-cultures of human Umbilical Cord MSCs (hUCMSCs) and cancer stem cells (CSCs) isolated from MCF-7 and MDA-MB-231 cell lines, reported a decrease in proliferation and augmented apoptosis of the latter *in vitro*. Also, when hUCMSCs were subcutaneously injected onto pre-established tumour bearing mice, a significant reduction in both size and weight of tumours was observed. MSCs tumour inhibition was sustained through a decrease in the levels of PI3K and Akt proteins, known to be involved in cancer development (79).

Interestingly, the methodology concerning MSC use in cancer studies may have some impact. The use of cells *per se* seems to have an associated risk, with possible differentiation into CAFs as reported above, for instance (62,80). Additionally, hypoxia conditions characteristic of the tumours have also been showed to stimulate MSC secretion of factors that inhibit tumour cell apoptosis and increase its proliferation, while normal MSCs did not took on these properties (23,81). On the contrary, an overall anti-tumoral effect of MSCs is herein observed when conditioned media is administered to tumour cells, thus possibly pending the balance for the investment in cell free based therapies. Therefore, it is crucial to understand the mechanisms related to this differential effect. Nevertheless, we can sum up some of the mechanisms sustaining MSCs cancer inhibition:

1) Promotion of apoptosis in cancer cells, both via upregulation of negative regulators of cell cycle such as p21, normally downregulated in cancer scenarios, and downregulation of the anti-apoptotic inhibitor, X-linked inhibitor of apoptosis protein (XIAP), usually upregulated in cancers, protecting cancer cells from apoptosis (82,83);
2) Tumour cell cycle arrest through secretion of a variety of cytokines, which results in impairment of tumour growth *in vivo* (82,84);
3) Disruption of cancer associated signalling pathways such as phosphoinositide 3-kinase (PI3K)/AKT, via upregulation of phosphatase and tensin homologue (PTEN), and of WNT/β-catenin signalling pathway by MSCs-secreted dickkopf 1 (DKK-1) factor, antagonist of the Wnt signalling, which ultimately leads to suppression of migration, invasion, and survival of tumour cells (75,85–87).

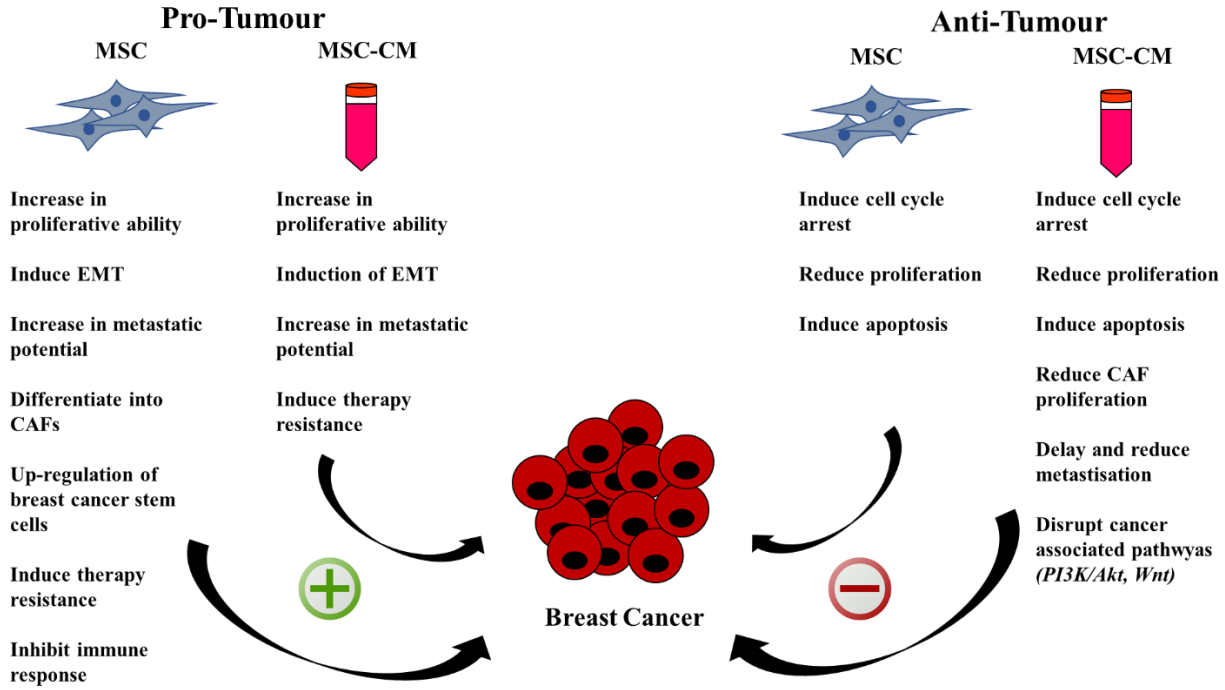

Figure 1.2. – Mesenchymal Stem Cells (MSCs) mediated effects in tumour progression and inhibition.

2. Objectives

This master thesis intends to shed light over the yet much controversial role of MSCs towards cancer therapy. The MSCs herein used, UCX® (ECBio, PCT/IB08/54067), are proprietary umbilical cord tissue-derived human neonatal MSCs (hnMSCs), whose isolation method has been fully adapted according to advanced therapy medicinal product (ATMP) requirements (88). These cells, being isolated from a neo-natal tissue, besides dodging the epigenetic alterations inherent to adult cells, also present an easier, inexpensive, less invasive, noncontroversial source of MSCs (1).

In an attempt to recreate the complex microenvironment of living systems, a prototypical 3D culture cell model, the spinner flask suspension culture (SFSC), was used, since that it better mimics the *in vivo* cell interactions. In this type of culture, cells aggregate/self-assemble into spheroid-like structures, enabling greater cell-cell and cell-matrix interactions, enhancing both cell expansion and differentiation, as well as allowing up-scaling processes.

In this context, the aim of this work was to uncover the role of the secretome of UCX® on breast cancer cells. To accomplish this, MSCs were cultured in 2D and 3D conditions, from which the MSCs secretome was collected. Afterwards, its effect on cell viability was accessed solely, and in combination with the chemotherapeutic agent dox in two different types of human mammary cells: a non-malignant (MCF10A) and a highly invasive carcinogenic (MDA-MB-231). Finally, migration and invasion ability of breast cancer cells was also assessed in the same experimental conditions.

3. Materials and methods

3.1. Chemicals

A 800 µM stock solution of Dox (D1515 – Doxorubicin hydrochloride from Sigma-Aldrich®) was prepared in H_2O MilliQ, from which working solutions were made.

3.2. UCX® culture

UCX® are a population of umbilical cord tissue-derived human neonatal mesenchymal stromal cells that were isolated as described in the patent WO/2009/044379 entitled of "Optimized and defined method for isolation and preservation of precursor cells from human umbilical cord" and was developed by ECBio, S.A (88) .

UCX® were cultured in UCX® medium consisting of α-MEM with L-glutamine and 1 g/L glucose (Minimum Essential Medium Eagle Alpha Modification, M0644 from Sigma-Aldrich®) supplemented with 10% of FBS (Heat inactivated Fetal Bovine Serum, 10500-064 from Gibco®) and 2.2 g/L of sodium bicarbonate (S5761 from Sigma-Aldrich®) in a humidified chamber at 37 ºC in a 5% CO_2 in air atmosphere.

For trypsinization purposes, cells were briefly washed with PBS (Phosphate Buffered Saline), and then incubated for 5 minutes in a 0.05% Trypsin-EDTA solution (25300-062 from Gibco®). Cells were then counted and cell viability was determined using the trypan blue (T8154 from Sigma-Aldrich®) exclusion method, under an Olympus CK30 inverted microscope.

In two dimensional monolayer cultures (2D), UCX® were seeded at a density of $1x10^4$ cells/cm^2 and incubated in a humidified chamber at 37 ºC in a 5% CO_2 in air atmosphere. Passages were made when cells reached 70-80% confluence.

Three-dimensional (3D) cultures of MSCs were performed through the use of spinner flasks, dynamic cultures which allow for a large-scale production of spheroids, promoting an enhanced biological function (22). For this purpose, cell suspensions were inoculated at a concentration of $1x10^6$ cells/mL in 125 mL spinner vessels with ball impeller. To stimulate the formation of spheroids, at day 0 cells were inoculated in the spinner flask in UCX® medium supplemented with 15% of FBS, stirred at 80-90 rpm and kept in a humidified chamber at 37 ºC in a 5% CO_2 atmosphere. After a period of 24 h, at day 1, FBS concentration was adjusted to 10%, and the medium was replaced every 3-4 days to allow nutrient availability as well as to decrease toxic by-products of cellular metabolism. During culture time, stirring rate was adjusted in order to avoid spheroids with more than 350 µm of diameter, thus preventing the formation of necrotic centres.

3.3. Production of UCX® Conditioned Medium

For the production of conditioned medium under 2D cultures (CM2D), at day 0 cells were passed and seeded at an inoculum of $1x10^4$ cells/cm^2 in 175cm^2 t-flasks and maintained in UCX® medium supplemented with 5% FBS, until reaching 90% of confluence, generally at day 3. At that point, cells were carefully washed with fresh α-MEM and medium was replaced by α-MEM without FBS, at a final volume of 25 mL per flask. After a 48 h conditioning period, at day 5, CM2D was collected under sterile conditions.

Conditioned medium from 3D cultures (CM3D) was obtained through the inoculation of cells in agreement with the protocol described above. After 24h, at day 1, FBS concentration was reduced to 5%, and cells were maintained in these conditions for 3 days. At day 4, medium was replaced by α-MEM without FBS. After a 48 h conditioning period, at day 6, CM3D was then collected under sterile conditions.

The control for CM2D and CM3D consisted of α-MEM with L-glutamine and 1 g/L glucose supplemented with 2.2 g/L of sodium bicarbonate without FBS, which was never in contact with cells.

Both CM2D and CM3D samples were concentrated 20x in 3kDa cut-off centrifugal concentrators (UFC900324 from Millipore®) as per manufacturer's recommendations. All samples were stored aseptically at -80 ºC until further use.

3.4. Culture of Human Mammary Cells

Human malignant breast carcinoma cell line MDA-MB-231 (ATCC) was maintained in DMEM with L-glutamine and 1 g/L glucose (Dulbecco's modified Eagle's medium, D5523 from Sigma-Aldrich®) supplemented with 10% FBS and 3.7 g/L of sodium bicarbonate (S5761 from Sigma-Aldrich®) in a humidified chamber at 37 ºC in a 5% CO_2 in air atmosphere. Trypsinization procedure was the same as described for UCX cells abovementioned.

MDA-MB-231 cells were usually seeded at a density of $2x10^4$ cells/cm^2 and incubated in a humidified chamber at 37 ºC in a 5% CO_2 in air atmosphere. Passages were performed when reaching around 70-80% of cell confluence.

Human mammary gland cell line MCF10A (ATCC) was maintained in DMEM/F12 medium (1:1 Dulbecco's modified Eagle's medium, D5523 from Sigma-Aldrich®:Nutrient Mixture F-12 Ham

medium, N3520 from Sigma-Aldrich®) containing 5% Horse Serum, 100 U/mL penicillin, 0.1 mg/mL streptomycin, 0.01 mg/mL insulin, 0.5 µg/mL hydrocortisone, 0.1 µg/mL cholera toxin, and 20 ng/mL human epidermal growth factor. Trypsinization consisted in briefly rinsing cells with PBS, followed by incubation for 10 minutes in 0.025% Trypsin-EDTA solution (25200-072 from Gibco®). Cells were counted and cell viability was determined through the abovementioned trypan blue method.

MCF10A cells were usually seeded at a density of $2x10^4$ cells/cm^2 and incubated in a humidified chamber at 37 ºC in a 5% CO_2 in air atmosphere. Passages were made when cells reached around 70-80% confluence.

3.5. Cell Viability Assay

The cytotoxicity of CM2D and CM3D, alone and in combination with dox, was evaluated through CellTiter 96® AQueous One Solution Cell Proliferation (MTS) Assay (Promega) in both MDA-MB-231 and MCF10A breast cell lines. This tetrazolium-based reagent is reduced by the cells, forming formazan crystals that are soluble in the cell culture medium, dispensing the use of a solubilization solution, which is usually required in other related viability assays such as the MTT. At death, cells rapidly lose the ability to reduce tetrazolium products, and therefore the production of the resulting coloured formazan product is proportional to the number of viable cells in culture.

For this purpose, $6.5x10^3$ MDA-MB-231 and $4.0x10^3$ MCF10A cells were seeded in 96-well plates in complete culture medium, for 48 h in a humidified chamber at 37 ºC in a 5% CO_2 atmosphere.

After 48 h of incubation, for the combined treatment of CM and dox, cell culture medium was replaced by 100 µL of each conditioned media (CM2D and CM3D) and 5 µL of dox (working solutions at 4, 10, 20 and 40 µM), both in complete cell culture medium in a total volume of 200 µL. Treatment with conditioned medium alone consisted of 100 µL of CM2D/CM3D and 5 µL of MilliQ H_2O (solvent for dox), in complete cell culture medium in a total of 200 µL. As for the treatment with dox without CM, this consisted of 5 µL of the drug and 100 µL of α-MEM (solvent for both CM2D and CM3D) in complete cell culture medium in a total of 200 µL. The final concentrations of CM2D and CM3D used were 1x and 10x concentrated, while for dox, final concentrations used were 100, 250, 500 and 1000 nM. For the control, cells were incubated in 100 µL of α-MEM and 5 µL of H_2O MilliQ in complete cell culture medium in a total of 200 µL. After another 48 h of incubation, medium was carefully discarded and 20 µL of CellTiter 96® AQueous One Solution Cell Proliferation Assay solution (MTS) were then added to the wells with 100 µL of cell culture medium without serum, and incubated at 37 ºC. After 1 h, absorbance was recorded by spectrophotometry at 490 nm in a SPECTROstar Omega multiplate reader (SPECTROstar Omega, BMG LABTECH).

Results were expressed as percentage relative to control, which was considered to be 100% of cellular viability. Three independent assays were performed, each one comprising three replicates.

3.6. Migration assay

Breast cancer migration was performed through an *in vitro* scratch assay, performed in accordance with Liang *et al*. (89). Briefly, $2x10^5$ MDA-MB-231 cells were seeded in 24-well plates in complete culture medium. After 24 h of incubation, the medium was discarded and a scratch was performed using a 200 µL pipette tip. Carefully, cells were then rinsed twice with PBS and test compounds were then added to the wells. Combined treatment of conditioned medium and dox consisted of 250 µL of CM2D/CM3D and 12.5 µL of the drug in serum free cell culture medium in a total of 500 µL. Treatment with conditioned medium alone consisted of 250 µL of CM2D/CM3D and 12.5 µL of MilliQ H_2O (solvent

for dox) in serum free cell culture medium in a total of 500 µL. Dox treatment consisted of 250 µL of α-MEM (solvent for CM2D/CM3D) and 12.5 µL of the drug in serum free culture medium in a total of 500 µL. Control treatment consisted 250 µL of α-MEM and MilliQ H_2O in serum free cell culture medium. Final concentrations for CM2D/CM3D were 1x and 10x concentrated, while dox was tested at a concentration of 0.1 µM. Scratches were evaluated microscopically (Motic AE 2000 inverted microscope), and three images of each scratch were recorded using Moticam 2500 at defined time points: 0, 7, 20, 24 and 30 h. Non-invaded area was measured using Motic Images PLUS v2.0 software. Three independent assays were performed, each one comprising three replicates.

3.7. Invasion Assay

Screening of the invasion capacity of breast cancer cells was made in ECM-coated transwells in 24 well plates. Transwells inserts of 8.0 µm (353097 from Corning®) were coated with 50 µL of a 0.3 mg/mL Matrigel™ solution (356230 form Corning®) and incubated for 1 h at 37 °C. Afterwards, $1x10^5$ MDA-MB-231 cells were briefly seeded in the upper chamber in serum free medium containing the test compounds in a total of 200 µL, while the lower chamber contained the same compounds in cell culture medium supplemented with 10% FBS (chemoattractant) in a total of 500 µL. Compound concentrations were maintained from the migration assay aforementioned, namely concentrations of 1x and 10x for CM2D/CM3D and a 0.1 µM concentration for dox. The plate was then left incubating for a 24 h period at 37 °C in a 5% CO_2 atmosphere, after which the inserts were carefully removed. Cells in the upper part of the insert were removed using a cotton swab and the invading cells in the lower part of the inserts were fixed with 96% cold ethanol for 15 minutes at 4 ºC. Staining of cells was then performed with a 0.1% Crystal violet solution for 10 minutes at room temperature, and cells were then left to dry overnight at 4 ºC. In the following day, representative photos of each insert were taken using a Motic AE 2000 inverted microscope. Finally, the remaining cell attached dye was dissolved with a 1% acid acetic in ethanol solution, and optic density was measured at 595 nm wavelength in a SPECTROstar Omega multiplate reader (SPECTROstar Omega, BMG LABTECH), in order to quantify breast cancer cell invasion.

3.8. Statistical Analysis

Statistical analysis was performed through GraphPad Prism v5.0 software (La Jolla, CA, USA). Two-way ANOVA with Bonferroni post-test was performed for cell viability assays with three independent assays. ANOVA repeated measures tool with Turkey's multiple comparison post-test was used when there were values matching, for migration and invasion assays. Results are presented as means ± standard deviation (SD). Statistical significance was represented as probability (p) value *<0.05, **<0.01 and ***<0.001.

4. Results and Discussion

4.1. 3D Cultures of MSCs

The main purpose of this work was to assess the effect of the MSCs (UCX®) secretome in breast cancer, as well as to ascertain if there would be any changes in UCX® mediated effects when using the

secretome produced in 3D culture conditions (CM3D), against the one obtained from traditional monolayers (CM2D).

Thus, the first step consisted on producing conditioned media from 3D UCX® spheroids cultured in a spinner flask suspension system. This model has already been optimized in a previous work conducted in our laboratory (38). Hence, parameters involved in 3D UCX® production, namely the inoculum ($1x10^6$ cells/mL), the impeller type (ball instead of paddle) and the agitation rate (80-90 rpm) were incorporated in this thesis. Nevertheless, the culture was monitored, and aggregates size was registered to ensure that the serum reduction to 0% applied in the process of medium conditioning would not affect UCX® spheroids morphology. Spheroid dimensions were measured via phase contrast microscopy images obtained during the culture period of conditioned media production (Fig. 4.1.A). Spheroid diameter rounded 130±28 µm at day 2 of culture, increasing to 179±35 µm and 208±29 µm at days 4 and 6, respectively (Fig. 4.1.B), therefore avoiding the formation of necrotic cores, that are reported to appear in aggregates with 350 µm or more (90). At day 6, CM3D was then collected under sterile conditions, to be further used in the *in vitro* viability, migration and invasion assays.

Concomitantly, the UCX® secretome was also produced in traditional 2D monolayer cultures (Fig 4.2.). Herein, at day 0 UCX® were seeded at a $1x10^4$ cells/cm^2 in 175 cm^2 t-flasks in 5% FBS, until reaching around 90% confluency, usually at day 3. At this point, medium was discarded and replaced by serum free medium and left conditioning for a 48 h period. At day 5, the protocol was finalized, and CM2D was collected in the same sterile conditions as CM3D.

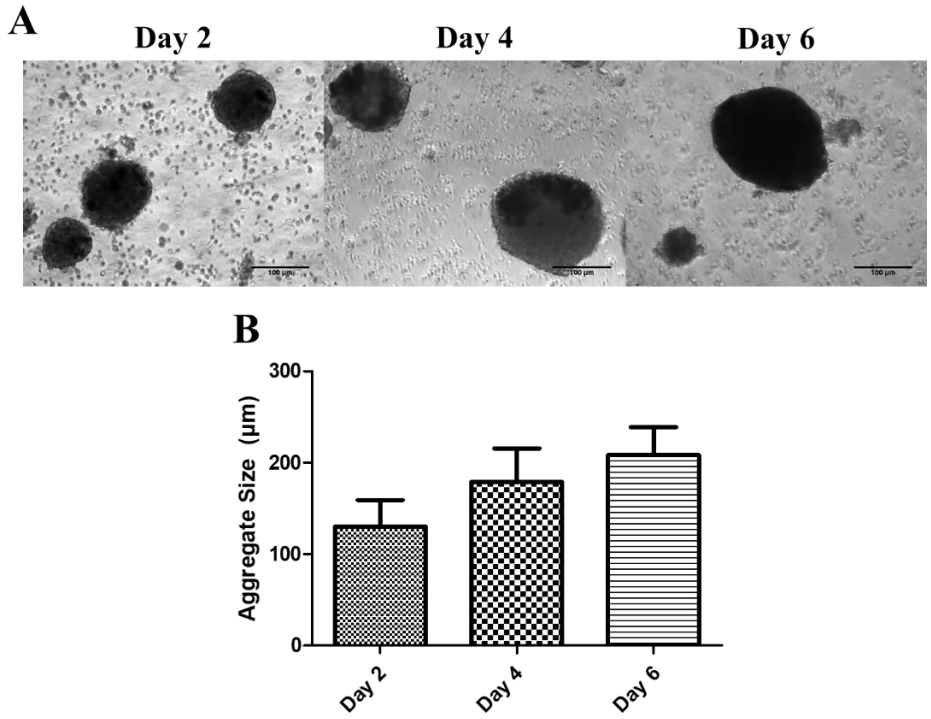

Figure 4.1. - UCX® spheroid formation in spinner flask suspension culture (SFSC) during CM production. **(A)** Representative images showing the aggregation of UCX® into spheroids cultured in SFSC with ball impeller at an inoculum of $1.0x10^6$ cells/mL; **(B)** UCX® spheroids diameter at days 2, 4 and 6. Values represent mean ± SD. Magnification 10x, scale bar = 100 µm

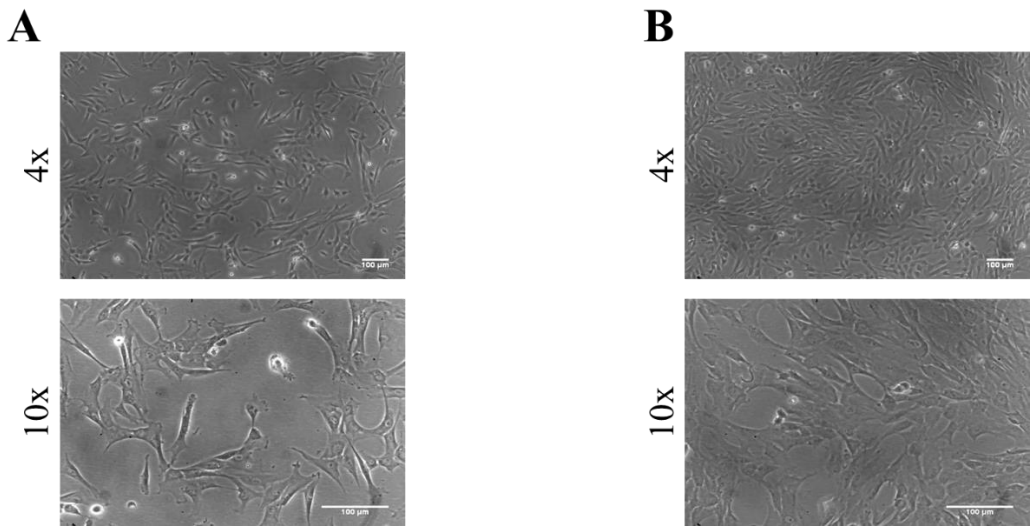

Figure 4.2. - UCX® cultured in traditional monolayer cultures (2D): (A) low cell confluency and **(B)** high cell confluency stage. Magnification 4 X, scale bar=100 μm; Magnification 10x, scale bar = 100 μm.

4.2. Cell Viability Assays

This work firstly aimed to evaluate the effect of the UCX® secretome on the viability of two breast cell lines: non-malignant breast epithelial MCF10A (Fig. 4.3.) and triple negative breast cancer MDA-MB-231 (Fig. 4.4.). Both cell lines were subjected to CM2D and CM3D in different concentrations (1x and 10x), alone or in combination with the chemotherapeutic agent dox (concentrations ranging from 100 to 1000 nM), as well as to the latter alone.

As shown in Figures 4.3. and 4.4., after a 48 h treatment, dox led to a concentration dependent inhibitory effect on cell proliferation. This effect was more marked in the case of the MCF10A non-carcinogenic cell line (approximately 14% of cell viability at 1000 nM of dox) when compared to the MDA-MB-231 breast cancer cell line (approximately 50% of cell viability at 1000 nM of dox).
In contrast, both CM2D 10x (Figure 4.3.A; $p<0.01$) and CM3D 1x and 10x (Figure 4.3.B; $p<0.01$) significantly enhanced proliferation on MCF10A cells when compared to control (non-treated cells). Concerning MDA-MB-231 breast cancer cells, only a slight increase of cell proliferation was observed, much less marked than the effect portrayed in the MCF10A cell line above, with no statistically significant differences obtained for the treatment with CM2D (Figure 4.4.A) or CM3D (Figure 4.4.B).
Remarkably, regarding the combined treatment with dox, both conditioned media displayed a trend for protection against the cytotoxicity of dox in the normal breast cell line. Herein, MCF10A cells treated with CM2D/CM3D and dox constantly displayed higher cell viability values when compared to cells treated with dox alone, especially for the 10x concentration in both media (Figure 4.3.). This protective effect was statistically significant for the dox concentration of 100 nM, more marked in CM3D (Figure 4.3.B; $p<0.001$) than CM2D (Figure 4.3.A; $p<0.05$), remaining however visible for dox concentrations up to 500 nM. Concerning the MDA-MB-231 cell line, conditioned media treatments did not display significant differences. However, a slight trend to promote cell proliferation and chemo-resistance was observed in the solo and combined treatment with dox, respectively (Fig. 4.4.).

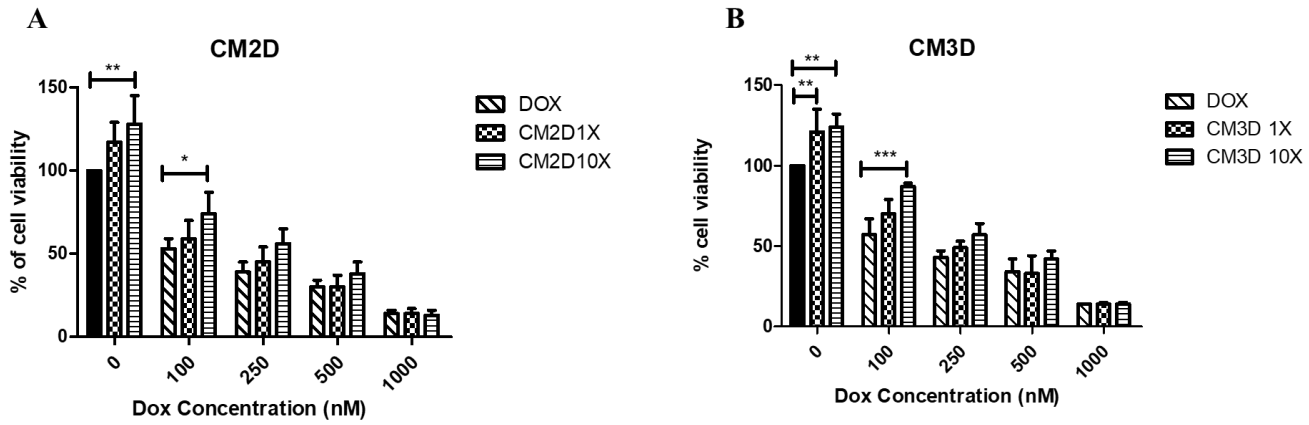

Figure 4.3. – Evaluation of conditioned medium derived from UCX® on MCF10A cell line viability: Evaluation though MTS assay of the effect of A) CM2D and B) CM3D alone or in combination with Dox (0.1-1 µM) on MCF10A cell viability: Results are expressed in percentage (mean ± SD, n=3) to control (black bar). Statistical significance is expressed relatively to control as *p<0.05, **p<0.01, ***p<0.001.

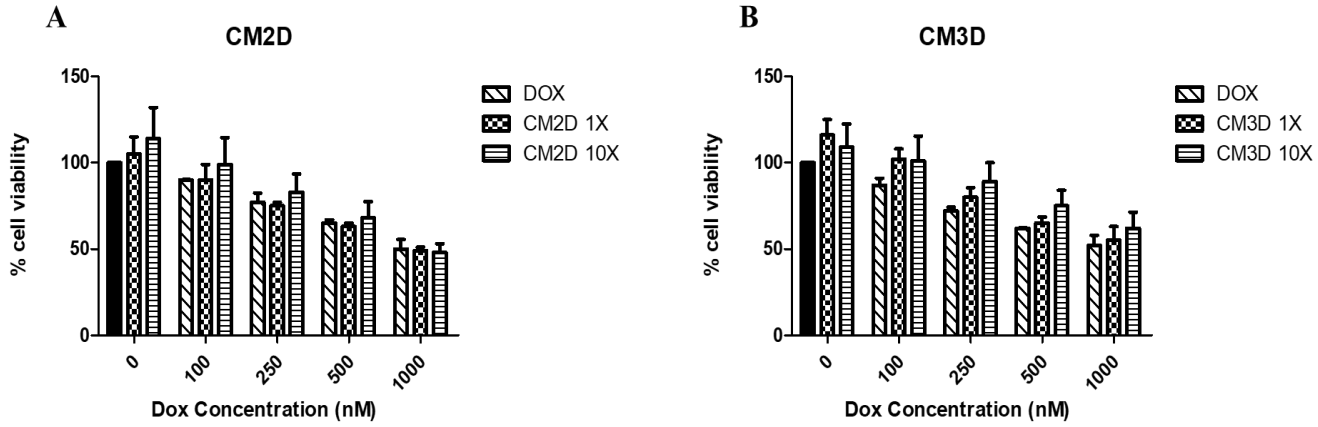

Figure 4.4. - Evaluation of conditioned medium derived from UCX® on MDA-MB-231 cell line viability: Evaluation though MTS assay of the effect of A) CM2D and B) CM3D alone or in combination with Dox (0.1-1 µM) on MDA-MB-231 cell viability: Results are expressed in percentage (mean ± SD, n=3) to control (black bar). Statistical significance is expressed relatively to control as *p<0.05, **p<0.01, ***p<0.001.

Dox inhibition of cell viability is in accordance with the literature, as it has been reported that this drug may interfere with the cell cycle, either by topoisomerase II inhibition, DNA intercalation, and free radical generation (91). Additionally, another study (92) showed that dox inhibition of cancer cells can yet be achieved via proteolytic cleavage of cAMP response element-binding protein 3-like 1 (CREB3L1), ultimately leading to the activation of the transcription of genes that encode cell cycle inhibitors, such as p21, and thus countering cancer cell hallmark number one, i.e. the sustain of proliferative signalling (93). Also, the less pronounced effect of dox in breast cancer cells when compared to the non-tumoral cell line in this study can be explained as cancer cells intrinsic resistance,

which poses a major challenge in cancer therapy. In fact, cancer cells are known to display greater resistance to drugs when compared to normal cells, mostly due to a subset of cells present within the tumour, named cancer stem cells (CSCs) (94). CSCs are known for presenting high tumorigenicity with an inherent ability to self-renew, and by dysregulating several pathways such as Wnt, Sonic Hedgehog and Notch signalling, displaying great resistance to radiation and chemotherapy, and playing an important role in the repopulation of the tumour after treatment (94).

Importantly, results of UCX®-CM treatment alone and in combination with dox showed a proliferative and protective effect of CM2D and CM3D in the non-tumoral breast cell line, respectively, being this effect more marked in the case of CM3D. The secretome profile of UCX® is in part previously known (38). In fact, UCX® possess a highly rich secretome, containing growth factors such as IL-6, FGF2, VEGF, KGF, with enhanced expression of almost all of the latter when cultured in 3D conditions (Fig. 4.5.). This was translated in enhanced wound healing after administration of CM3D into *in vivo* mice models leading to a faster and more effective therapeutic effect when compared to CM2D (38). In addition, a very interesting review published in 2015 regarding stem cells also states that, when cultured in 3D conditions, cells display an enhanced expression of anti-apoptotic (Bcl-2) and lower expression of pro-apoptotic (BAX, caspase) factors when compared to cells in traditional monolayer cultures (22). Indeed, the presence of these factors in the secretome in study is a possible explanation for the enhanced proliferation and chemo-protection herein conferred by UCX®-CM to non-tumour cells, particularly in the case of CM3D.

In the breast cancer cell line, although not displaying any significant results, UCX®-CM alone and combined treatment with dox slightly increased cell proliferation and protection against the drug, respectively (Fig. 4.4.). A similar yet more marked effect as the one from the solo treatment was described by Li et al., in which administration of UC-MSCs conditioned medium (10% and 20% in cell culture medium) led to a significant increase in proliferation of MCF-7 and MDA-MB-231 cell lines *in vitro*. This was correlated to an up-regulation of the ERK pathway, thus the modulation of this pathway may as well be in the basis of the UCX®-CM alone treatment on cancer cells in this thesis, although in a less marked mode (95).
Moreover, MSC slight chemo-protection over MDA-MB-231 cells against dox herein observed has also been previously reported. In this matter, a study in which MDA-MB-231 cells were exposed to adipose tissue (AT)-MSCs CM, a decrease in intracellular accumulation of dox was reported, extensively increasing chemo-resistance, due to an up-regulation of breast cancer resistance protein (BCRP). BCRP is an adenosine triphosphate-dependent membrane transporter that can drive substances across membranes against the concentration gradient. Therefore, by up-regulating BCRP, AT-MSCs CM stimulated a greater efflux of dox from cancer cells. Importantly, IL-8 secreted by MSCs was established as the principal responsible for this outcome (96). Although its presence on the UCX® secretome has not been confirmed, as only a few factors were there approached in the context of wound healing (Fig. 4.5.), IL-8 may also be involved in this process. Difference in the magnitude of the chemo-protection reported in the BCRP study versus the one from this thesis may come from the different source of MSCs used. The slight UCX® protective trend in MDA-MB-231 cells is depicted for dox concentrations up to 1000 nM for CM3D 10x, while for CM2D 10x this is only visible up to 500 nM concentration of the drug (Fig. 4.4.). In fact, as was mentioned in the "3D cultures" section of the introduction, IL-8 factor is up-regulated in these conditions, which may help to explain the enhanced protective effect depicted for CM3D versus CM2D (22). Additionally, the context of overall increased anti-apoptotic factors and decreased pro-apoptotic factors in 3D cultures mentioned as a possible explanation for the protective effect in the non-tumour cell line may also be herein applied to the breast cancer cell line, in a lesser magnitude.

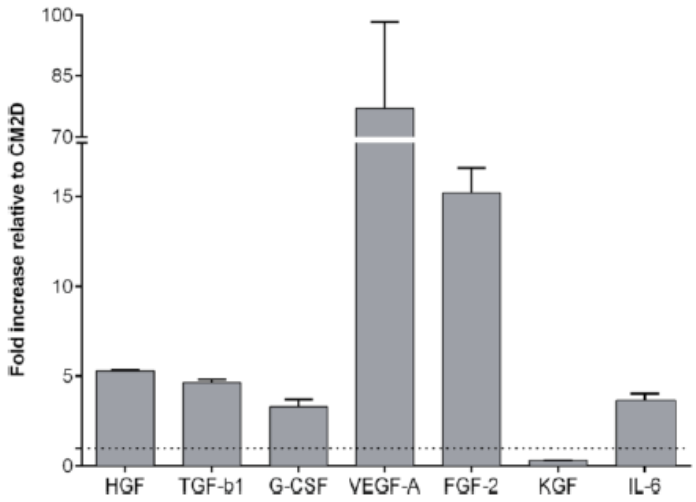

Figure 4.5. - Quantification of UCX®-MSCs secreted growth factors in spheroid (CM3D) cultures versus traditional monolayer cultures (CM2D). Adapted from Santos et al. Stem Cell Research & Therapy, 2015.

4.3. Migration Assay

To further evaluate the safety in the possible use of UCX®-CM in cancer therapy, the next objective of this study was to evaluate in what extent the latter would affect the migratory capacity of the breast cancer cell line MDA-MB-231 *in vitro*. Thus, cells were treated with both CM2D and CM3D 1x and 10x concentrated, alone and in combination with dox at 100 nM in an *in vitro* scratch wound healing assay. The 100 nM concentration of dox was chosen as it did not show a significant cytotoxic effect in the breast cancer cell line in the previous viability assay conditions. The migration assay was made in serum free conditions, to ensure that the closing of the scratch was accomplished via cell migration, and not cell proliferation.

24 h after scratch performance, a significant increase in wound closure was observed for cells treated either with CM2D and CM3D at a 10x concentration when compared to the control ($p<0,05$) (Fig. 4.6.A and B). At a 1x concentration, CM2D displayed a similar effect to the control (non-treated cells), while CM3D was able to stimulate cell migration ~2 fold when compared to control, although not in the extent of the 10x concentration.
Dox alone displayed a similar effect to CM2D 1x, i.e., non-significant differences were observed relative to control. This was also noted in the combined treatment, where dox did not influence the effect of the conditioned medium overall.
Three other time points were also analysed, at 7 h, 20 h and 30 h (Figures 4.8.). 7 h after performing the scratch, it was possible to observe a faster closing of the wound in the case of CM3D 10x ($p<0.05$) and for CM3D 1x with dox ($p<0.05$), as well as of CM2D 10x, although in a slighter way (n.s.), when compared to controls (non-treated). The major increase in the scratch closure was observed between the 7 h and 20 h time points, being less pronounced between the 20 h and the final 30 h time points. Between these two last time points, the evolution of the scratch observed is in accordance to what was described for the 24 h time point.

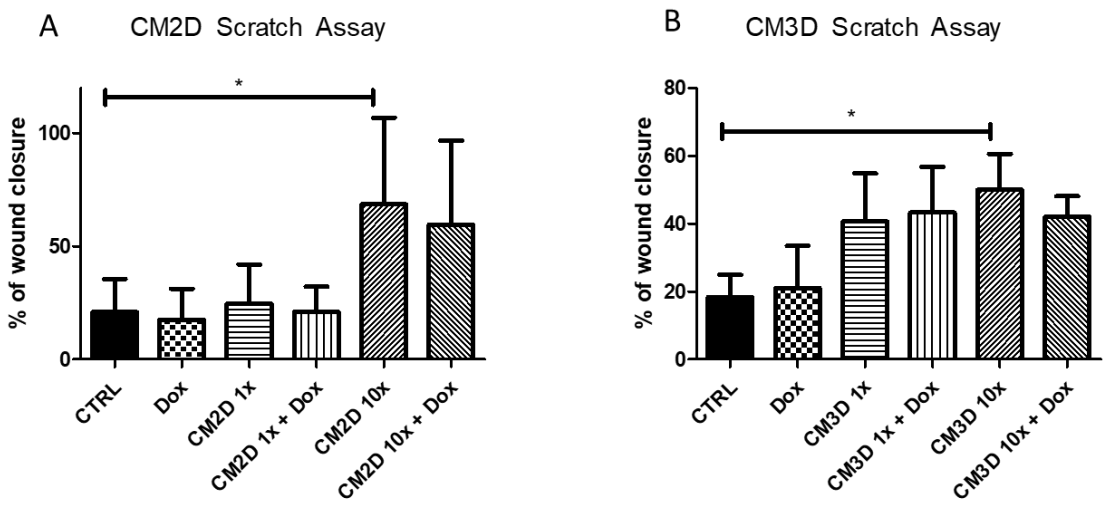

Figure 4.6. – Evaluation of the conditioned medium derived from UCX® on MDA-MB-231 cell line migration ability (24h): Effect of A) CM2D and B) CM3D alone or in combination with Dox (0.1 µM) on MDA-MB-231 cell migration at 24 hours: Cells migration represented as percentage (mean ± SD) of wound closing after 24 hours. Statistical significance is expressed relatively to control as *p<0.05, **p<0.01, ***p<0.001.

Overall, the results obtained in this assay show that UCX® CM enhanced the migratory ability of breast cancer cells ($p<0.05$ for CM2D 10x and CM3D 10x). The mechanism behind the UCX® pro-migration effect here observed may be approached as result of induction of Epithelial to Mesenchymal Transition (EMT) in cancer cells. EMT is a natural and important process in several organism phenomena such as embryonic development, organogenesis and wound healing, but usually suffers aberrancies in a cancer context, that can also contribute to therapeutic resistance and increased breast cancer cell motility (97). In fact, UC-MSCs conditioned medium has already been reported as an EMT promotor in breast cancer MDA-MB-231 cells (95). In this extent, through activation of the ERK pathway, UC-MSCs CM inhibition of E-cadherin and overexpression of EMT associated factors N-cadherin and ZEB1 were observed, resulting in an enhancement in breast cancer cell migration ability in an *in vitro* scratch assay, similarly to what is described in the present thesis, and in a transwell assay. Therefore, one can hypothesise the involvement of ERK pathway in UCX® promotion of cancer cell migration noticed herein, by EMT activation.

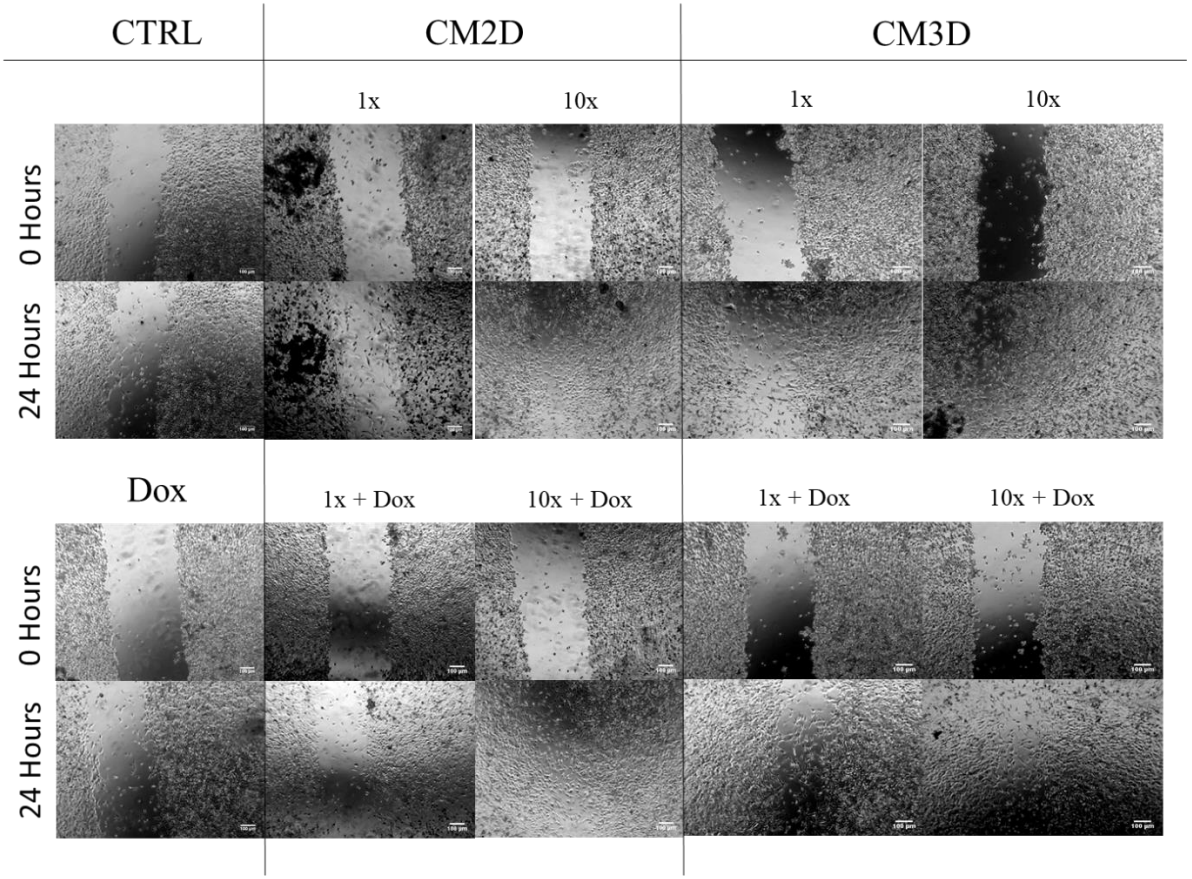

Figure 4.7. - Representative images of scratch assays at 0 and 24 hours with teste compounds and control. Magnification 4 X, scale bar=100 μm.

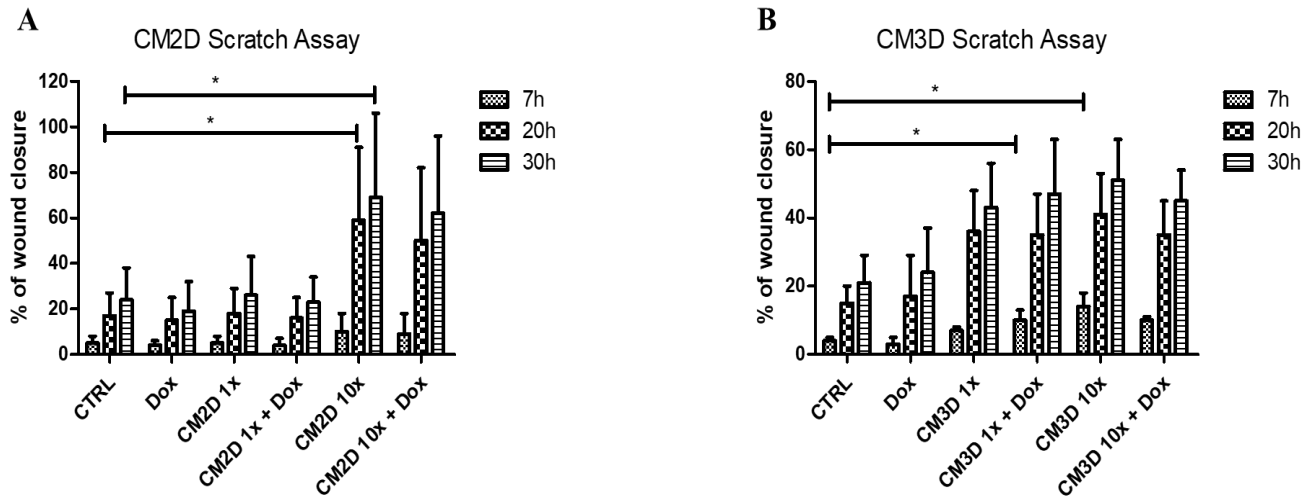

Figure 4.8. – Evaluation of the conditioned medium derived from UCX® on MDA-MB-231 cell line migration ability (7, 20 and 30h) Effect of (A) CM2D and (B) CM3D alone or in combination with Dox (0.1 μM) on MDA-MB-231 cell migration at A) 7 hours, B) 20 hours and C) 30 hours: Cell migration represented as percentage (mean ± SD) wound closing at the defined time points. Statistical significance is expressed relatively to control as *p<0.05, **p<0.01, ***p<0.001.

Of importance, TGF-β factor (present in UCX® secretome – Fig. 4.5.) is also known to be implicated in EMT of epithelial cells, being its expression abnormally upregulated in cancers by tumour stromal

cells, inducing cancer cell EMT (97). In a study where MSCs were primed with TNF-α and IFN-γ, pro inflammatory factors in order to simulate the tumour microenvironment, induction of EMT in breast cancer MCF-7 cells and pancreatic adenocarcinoma (PANC-1 and BxPC-3) cell lines was reported via TGF-β up-regulation (98). Additionally, IL-6 and HGF factors also present in UCX® secretome were also able to promote EMT respectively in gynecological cancer cells (GCC) and in luminal mammary progenitors, thought to be in the origin of triple negative breast cancer, like the one approached in this thesis (99,100).
Thus, the already known UCX® factors TGF-β, IL-6 and HGF may have had also some influence in the enhancing of breast cancer cell migration herein observed.
Nevertheless, further studies are indeed needed to clarify the mechanism behind these results.

4.4. Invasion Assay

Finally, the effect of UCX®-CM on MDA-MB-231 cells ability to invade was also evaluated, being one of the most important features towards a metastisation outcome. This is accomplished through a cascade of biological processes, including loss of cell adhesion, increased motility and invasion (101). The same concentrations were adopted, namely CM2D/3D 1x and 10x, and dox at a 100 nM concentration.

Herein, through a coated transwell assay, UCX®-MSCs CM did not show any significant effect over MDA-MB-231 invasion capacities *in vitro*. Particularly, the cells treated with dox alone seemed to slightly decrease cell invasion (~7% - Fig. 4.9.A and B) when compared to control (non-treated) (n.s.). Interestingly, it is possible to observe some opposing treatment-related trends in the case of CM: when administrated solely, CM2D 10x (Fig. 4.9.A) and CM3D 1x and 10x (Fig. 4.9.B) seemed to favour a slight increase in cell invasion when compared to controls (16% for CM2D 10x; 8% and 16% for CM3D 1x and 10x, respectively); while combined treatment of dox with CM 10x slightly reduced cells invasion in 10%/16% (2D – Fig. 4.9.A) and 6%/15% (3D – Fig. 4.9.B), when compared to dox alone and control, respectively.

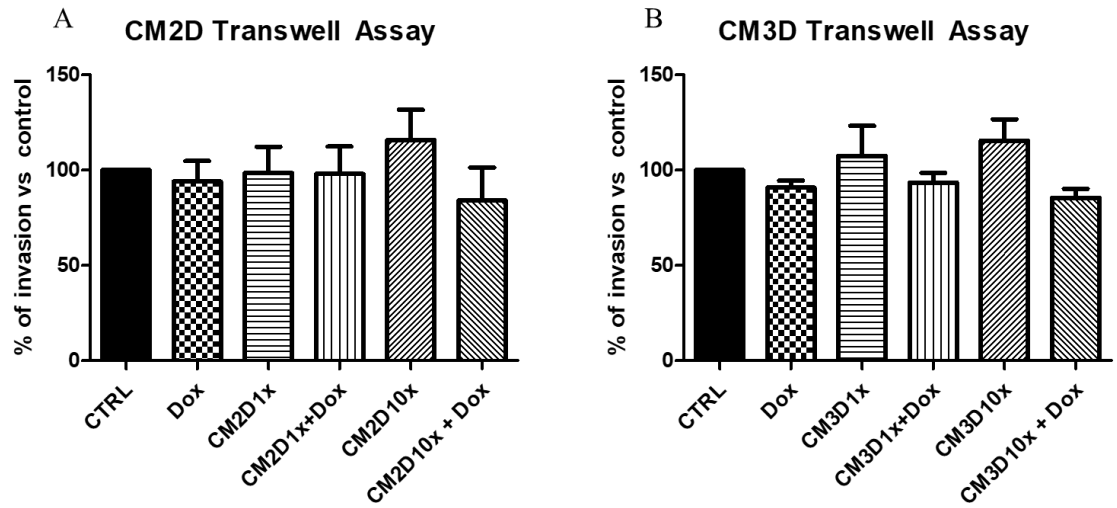

Figure 4.9. – Evaluation of the conditioned medium derived from UCX® on MDA-MB-231 cell line invasion ability: Effect of A) CM2D and B) CM3D alone or in combination with Dox (0.1 µM) on MDA-MB-231 cell invasion: Results are expressed in percentage (mean ± SD, n=4) to control.

Overall, the slight trend to increase cell invasion depicted for the CM solo treatment can also be correlated with promotion of EMT, as was mentioned in the migration section above, seeing that this process also plays a central role in the development of tumour metastasis (97). Moreover, capacity of MSCs to promote tumour metastasis was already reported in the literature, as BM-MSCs effectively promoted invasion and facilitated the metastatic spread in *in vivo* models of osteosarcoma and breast cancer, in a mechanism based on the secretion of CCL5 chemokine (61,102). Thus, as the expression of EMT promoting UCX® factors TGF-β, IL-6 and HGF mentioned in the Migration section above may also explain the results herein obtained, others potentially not yet uncovered, such CCL5, may also be here implied, although further studies are needed for confirmation.

On the other hand, the combined treatment of CM and dox pointed out to a reduction of breast cancer cell invasion, which is in agreement with a study by Fernandes et al (103). In that study, this was attributed to a pro-oxidant effect by dox, leading to augmented intracellular levels of reactive oxygen species (ROS), in a mechanism proposed to be modulated by H_2O_2 signalling (103). Therefore, one could hypothesize that the combined treatment's trend to reduce cell invasion may also be mediated by an increase in expression of ROS induced by dox, and this mechanism can be facilitated by the presence of the CM due to ROS modulation. This has yet to be proved, though, as other mechanisms may be here taking place.

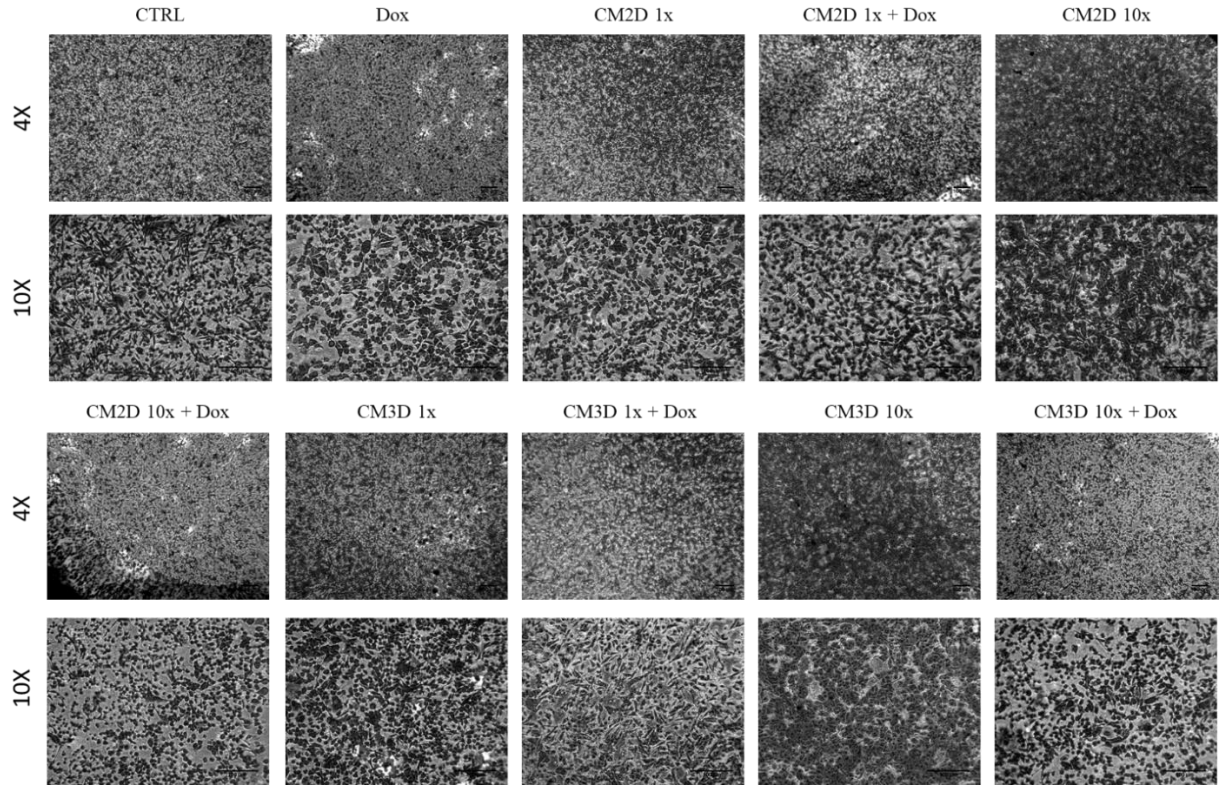

Figure 4.10. - Representative images of transwell assay with test compounds and control. Magnification 4 X, scale bar=100 μm; Magnification 10x, scale bar 100 μm.

5. Conclusion

This work studied the effect of the conditioned media produced from UCX® cultured in 2D and in 3D conditions in a breast cancer context. Importantly, the combined effect of MSCs with chemotherapeutics was a novel approach made in this study, as it had never been reported before in the context of protecting non-tumour cells. Moreover, even regarding cancer cells, the fact is that most literature reports focus on MSCs isolated action.

Herein, remarkably, viability assays showed that the UCX® secretome effectively protected non-tumour breast cells from the cytotoxic action of dox, while barely affecting the proliferation of breast cancer cells themselves, a promising result towards the protection of normal tissues adjacent to tumours. Additionally, 3D cultures also showed a more potent paracrine activity, as the aforementioned protective role of UCX® secretome on non-tumour cells was greater in the form of CM3D. Also, contrarily to migration assays, the combination of CM and dox showed a tendency to reduce breast cancer cell invasion.

The type of experiment methodologies can serve as a possible explanation for the differential results among migration and invasion. If on one hand the scratch wound healing assay provides insight over cells' motile ability as a collective unit, the chemotaxis (transwell - invasion) assay evaluates the motility of individual cells (103), and thus it would be interesting to complement the scratch assay with a transwell uncoated assay, to test breast cancer cells migration capacity as isolated units.

As such, the opposite directions obtained in the viability, migration and invasion sections of this thesis should be clarified in future studies by studying the mechanisms behind UCX® protection of non-tumour cells against dox, and on the other hand, understanding which sustains the increased breast cancer cell migration observed. Likewise, it would be very interesting to understand the exact mechanism sustaining the trend for reducing invasion showed by the combined treatment in the invasion assays, in the prospect of future combined therapies for cancer.

Additionally, dox greatest clinical risk is related to its induced cardiotoxicity (54). Therefore, and having in account the protective role displayed by UCX®-CM in normal epithelial breast cells against this drug, it would be very interesting to try and translate this to a model of the heart, again in the perspective of reducing the side effects of chemotherapy. It would be also interesting to approach 3D cultures of MSCs for this matter.

6. References

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